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Quantitative In Vitro Assay for "Corncob" Formation

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The interaction of *Bacterionema matruchotii* with strains of *Streptococcus sanguis* produces a structure which morphologically resembles a corncob. To determine the specific bacterial surface receptors involved in the interaction, we developed a quantitative assay. The assay consisted of mixing saline suspensions of [CH_3-^{3}H]thymidine-labeled streptococci and *B. matruchotii*, incubating at 37°C for 2 h, and filtering the mixture through a 5-μm polycarbonate membrane filter. The free cocci and filaments passed through the filter, but the corncobs were retained. Estimates of the number of corncobs formed were obtained by quantitating the radioactivity retained on the membranes relative to that of controls of streptococci alone. Although saturation of the *Bacterionema* occurred at a ratio of streptococci to *Bacterionema* of 10:1 (Klett units), a 2:1 ratio was chosen because of the increased sensitivity of the assay at this ratio. The percentage of streptococci binding at this ratio was 18.6 ± 8.1 (standard deviation). All five *Bacterionema* strains tested formed corncobs; in contrast, only three strains of *S. sanguis* were positive. These were serotype 1 strains which had localized surface "fuzz." Although scanning electron microscopic observations revealed an almost random distribution of cocci along the filament surface, transmission electron microscopy revealed that the streptococci were attached to the *Bacterionema* by the surface fuzz. No differences in corncob formation were observed in sodium phosphate buffer, pH 6 to 8, at phosphate concentrations ranging from 0.005 to 0.05 M. Concentrations of NaCl or KCl up to 0.25 M did not affect corncob formation, and low concentrations of $CaCl_2$ increased corncob formation slightly, whereas $MgCl_2$, ethylenediaminetetraacetic acid, and citrate buffers reduced the number of streptococci binding to the filaments. These results suggest that divalent cations may play a role in this process.

Dental plaque is an accumulation of oral bacteria in an adherent matrix of polysaccharide and other bacterial and host products on the teeth and appears to be a precursor of both dental caries and periodontal disease. Although the quantitative microbial composition of plaque is highly variable, it does show evolutionary succession with increasing age. As plaque matures, the initial predominantly gram-positive coccal flora becomes "invaded" by a mixture of gram-positive rods, gram-negative cocci, and filaments (18, 27). *Streptococcus sanguis* appears to be among the first organisms to colonize the tooth surface (5–7), possibly because of the affinity of *S. sanguis* for the salivary glycoproteins in the acquired pellicle on the tooth surface (36). This organism may also interact specifically with other organisms found in mature plaque, such as *Actinomyces viscosus* (4, 8, 11, 20). Another interesting interaction between bacteria in plaque is the "corncobs," which were originally observed as morphological entities in the 19th century (37). The term corncob was coined to describe a morphological unit consisting of a filamentous organism surrounded by adherent cocci (14). These aggregates have been the subject of several recent investigations (15, 17, 22–24, 35). They are found in the superficial layers of supragingival plaque (18); the filamentous component has been identified as *Bacterionema matruchotii* (21). Two streptococcal isolates of the coccal component were obtained from in vivo corncobs obtained by micromanipulation (22) and identified as strains of *S. sanguis* (23). Takazoe and his co-workers have also identified five streptococcal strains which form corncobs with *B. matruchotii* and *S. sanguis* (35). The distinctive morphological features of the corncob configuration make it a convenient model for the study of the specificity of interbacterial aggregation. The objective of this initial study was to establish a quantitative, reproducible assay for the in vitro production of corncobs. Such an assay would form the basis of future studies

directed at determining whether specific surface receptors are involved in the formation of these aggregates as well as at characterizing these receptors.

MATERIALS AND METHODS

Strains, media, storage, and growth conditions. *S. sanguis* strains CC5A and CC6 were obtained from C. Mouton (State University of New York, Buffalo, N.Y.). *B. matruchotii* ATCC 14266 was obtained from the American Type Culture Collection, Rockville, Md.; *B. matruchotii* strains 18, 197, 208, and 214 were obtained from C. Mouton. Most of the other streptococcal strains used in this study were obtained from our culture collection and have been described in previous studies (1, 29). Strains H-24 and H-311 were obtained from Pauline Handley of the University of Manchester, Manchester, England (13). These two strains were isolated from dental abscesses and classified as *Streptococcus mitior* (*mitis*) (13); however, by criteria established in our laboratory (28, 30) and a modification of the biochemical classification of Facklam (10), they have been classified as *S. sanguis*, serotype 1. They possess microscopically demonstrable localized tufted fimbriae, or "fuzz" (13). The streptococcal cultures were grown in brain heart infusion broth (Difco Laboratories) at 37°C for 18 h; 1-ml samples were aseptically transferred to sterile vials, rapidly frozen in alcohol-dry ice, and stored at −70°C. Similar conditions were used for the *B. matruchotii* cultures, except incubation was with moderate shaking for 48 h, and 2-ml samples were removed for storage. These frozen culture samples were used as starter cultures for the studies described in this report. Thus, all of the studies reported here represent essentially the same initial cultures. These frozen starter cultures were thawed and inoculated into 1 liter of brain heart infusion broth, which was then incubated for 48 to 72 h before harvesting.

Culture harvesting. The cultures of streptococci and *Bacterionema* were harvested by centrifugation at 9,200 × *g* for 15 min at 4°C. The cell pellets were suspended in 1/10 of the original culture volume in cold 0.15 M NaCl and repelleted. This procedure was repeated once more, and the final pellet was resuspended in 0.15 M NaCl such that a 1:10 dilution gave a reading of 100 on a Klett colorimeter with a blue filter (470 nm). The suspensions were stored on ice for a maximum of 7 days.

Radioactive labeling conditions. Streptococci were labeled by growth for 48 to 72 h in brain heart infusion broth containing 0.1 μCi of [CH_3-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml and then harvested as described above. The time of incubation was selected on the basis of preliminary morphological studies which suggested that better corncobs were formed with older cells. The specific activity of the streptococci was 1 cpm per 4×10^5 cells.

Assay procedure. The reaction mixture consisted of 0.20 ml of *Bacterionema* suspension (200 Klett units; 2×10^7 cells per ml is equivalent to 1 Klett unit), 0.40 ml of the streptococcal suspension (400 Klett units), and 1.40 ml of NaCl in a plastic foam-stoppered 25-ml Erlenmeyer flask. The mixtures were incubated for a minimum of 2 h at 37°C in a New Brunswick rotary water bath shaker with moderate shaking (speed setting, 5). Two controls were used in each assay: (i) streptococci suspended in saline and (ii) streptococci suspended in the supernatant obtained by incubating the *Bacterionema* cells in NaCl at 37°C for 2 h. Substitution of vessels with different shapes such as scintillation vials reduced the number of corncobs formed. The presence of corncobs was qualitatively assayed by phase-contrast microscopy. The quantitative assay consisted of filtering the reaction mixture through a Nuclepore polycarbonate membrane (5-μm pore size; 25-mm diameter). The flask was rinsed three times with 2 ml of NaCl each, and the washings were filtered; the membrane was washed twice with 1 ml of saline. The washed membranes were placed in Aquasol (New England Nuclear Corp.) and counted in a liquid scintillation spectrometer. The controls of the streptococci in saline or incubated with the *Bacterionema* supernatant were also filtered, and the numbers of counts adhering to the filters were deducted from the experimental values. Counts of the cells in the filtrate were also performed, and recoveries of radioactivity from the filter plus the filtrate were approximately 90% of cell input.

Effects of pH and salts on corncob formation. NaCl, KCl, $MgCl_2$, $CaCl_2$, and ethylenediaminetetraacetic acid at concentrations of 0.05 to 0.25 M were tested to determine effects on corncob formation. Sodium phosphate buffer, pH 6.5, from 0.005 to 0.05 M with and without NaCl was also tested. The effects of pH from 6 to 8 were tested in 0.05 M phosphate buffer; citrate-phosphate buffer was used at a pH range from 4.5 to 8. Clarified whole saliva was obtained from a single donor by the procedure described by Appelbaum et al. (1) and tested for its effect on corncob formation.

Sonication. An MSE sonicator set at maximum output was used at 1-min intervals up to a total of 5 min in an ice bath to determine effects of sonication on corncob formation.

Electron microscopy. For transmission electron microscopy, cells were fixed in freshly prepared 2% (wt/vol) glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 (G. Millonig, J. Appl. Physiol. **32**:1637, abstr. no. 1326, 1961), for at least 1 h at 4°C. After enrobing in a small amount of warm (45°C) Noble agar, the cell pellets were washed four times in cold phosphate buffer and postfixed in 2% (wt/vol) osmium tetroxide for 1 h at 4°C. After osmium treatment, the pellets were washed once with phosphate buffer, dehydrated through a graded ethanol series, and embedded in Epon 812 (19). Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome; the sections were stained with uranyl acetate and lead citrate (26) and examined in a JOEL 100S transmission electron microscope.

Samples for scanning electron microscopy were prepared as described by Poirier et al. (25) and examined in a JEOL 25S scanning electron microscope.

RESULTS

Figure 1 shows the relationship of incubation time to the percentage of streptococci bound to the *Bacterionema*. The percent bound levels off

after 2 h of incubation, suggesting that saturation has occurred; thus, this incubation time was selected for the assay.

The optimum ratio of cocci to filaments was determined by varying the amount of cocci added to the reaction mixture at a constant *Bacterionema* concentration. As shown in Fig. 2, saturation occurs at a coccus/filament ratio of 10:1, expressed in Klett units. The coccus/filament ratio of 2:1 was chosen for future work, since this value is in the most rapidly changing portion of the curve and therefore provides optimal sensitivity for the assay.

The precision of the typical assay is high, as shown in Table 1, in which an estimation of corncob formation by three batches of cocci and filaments prepared over a 3-month period were compared. To date, 65 experiments which include those shown in Table 1 have been performed. The mean percentage of streptococci binding to the membranes in the blanks is 3.6 ± 2.5 (standard deviation); the mean percent streptococci binding to the *Bacterionema* is 18.6 ± 8.1%. The mean of the coefficients of variation in these 65 experiments was 0.127 ± 0.009.

Phase-contrast light micrographs of typical in vitro corncobs are shown in Fig. 3; the variety of different arrangements suggests a random distribution. Scanning electron micrographs (Fig. 4) confirm a random attachment of the streptococci to the *Bacterionema*. In most instances, the streptococci were observed to be arranged along the length of the filament, rather than at the filament termini. It is not clear from the microscopic observation whether there are specific locations or areas along the filament to which the streptococci adhere; similar observations were made by Bayer for bacteriophage adsorption (3). Thin sections of chemically fixed corncobs (Fig. 5A and B) and CC5A (Fig. 5C) show the presence of a localized electron-opaque fuzz on the cocci. This fuzz layer appeared to be involved in the adhesion of the cocci to the filaments; all of the strains of *S. sanguis* which formed corncobs had the localized fuzz associated with the surface of the *Bacterionema* cell wall.

The unusual nature of the *Bacterionema* cell wall is shown in detail in Fig. 5B. The inner cytoplasmic membrane is separated from the electron-opaque outer layer by the thick intermediate peptidoglycan layer similar to that

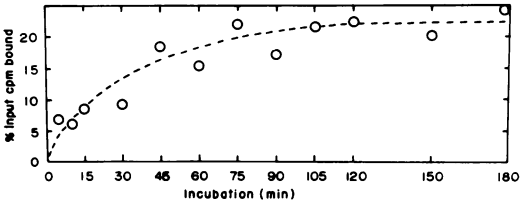

FIG. 1. *Radioactivity in counts per minute bound to Nuclepore polycarbonate membranes as a function of time of incubation.*

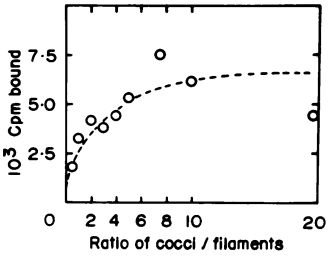

FIG. 2. *Radioactivity in counts per minute bound to Nuclepore membranes as a function of the coccus/filament ratio (expressed in Klett units).*

TABLE 1. *Comparison between typical corncob assays*

Assay	Input cpm	cpm bound to assay membrane	Mean	SD[a]	Cv[b]	Blank[c]	Corrected mean (cpm)[d]	Cocci in corncobs (%)	Cocci in blank (%)
1	18,162	3,288 3,742 3,879	3,636	309	0.085	100	3,536	19.5	0.55
2	16,208	4,837 4,340 3,537	4,245	645	0.152	425	3,820	23.6	2.60
3	21,123	5,223 5,250 5,348	5,274	66	0.012	528	4,746	22.5	2.50

[a] SD, Standard deviation.
[b] Cv, Coefficient of variation.
[c] Blank consisted of cocci incubated in saline extract of *Bacterionema* at 37°C.
[d] Counts per minute bound to membrane in assay minus the counts per minute in blank.

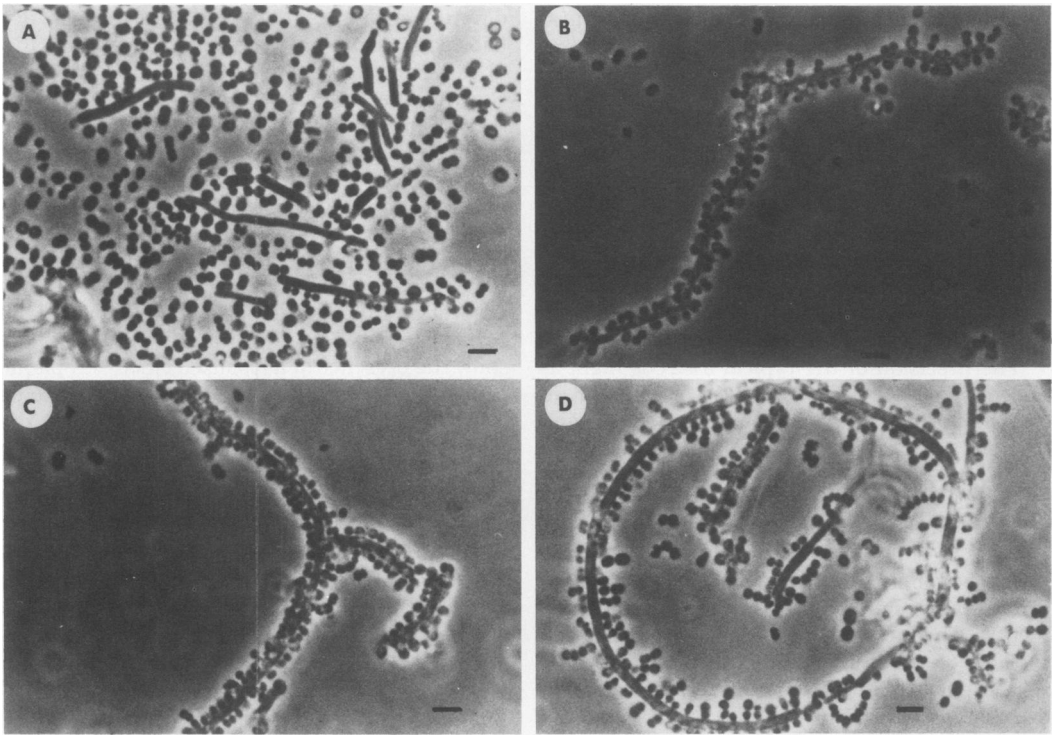

FIG. 3. *Phase-contrast light micrographs. (A) Typical assay filtrate; (B, C, and D) typical in vitro corncobs. Bar = 1 μm.*

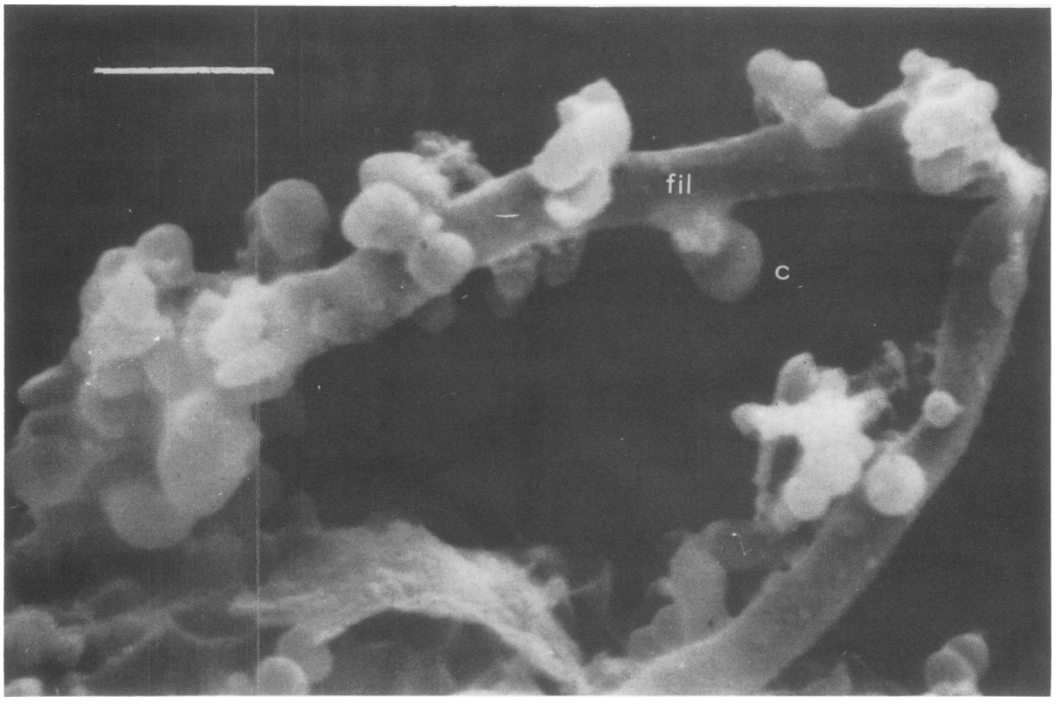

FIG. 4. *Scanning electron micrograph of an in vitro corncob. fil, Filament; c, cocci. Bar = 2 μm.*

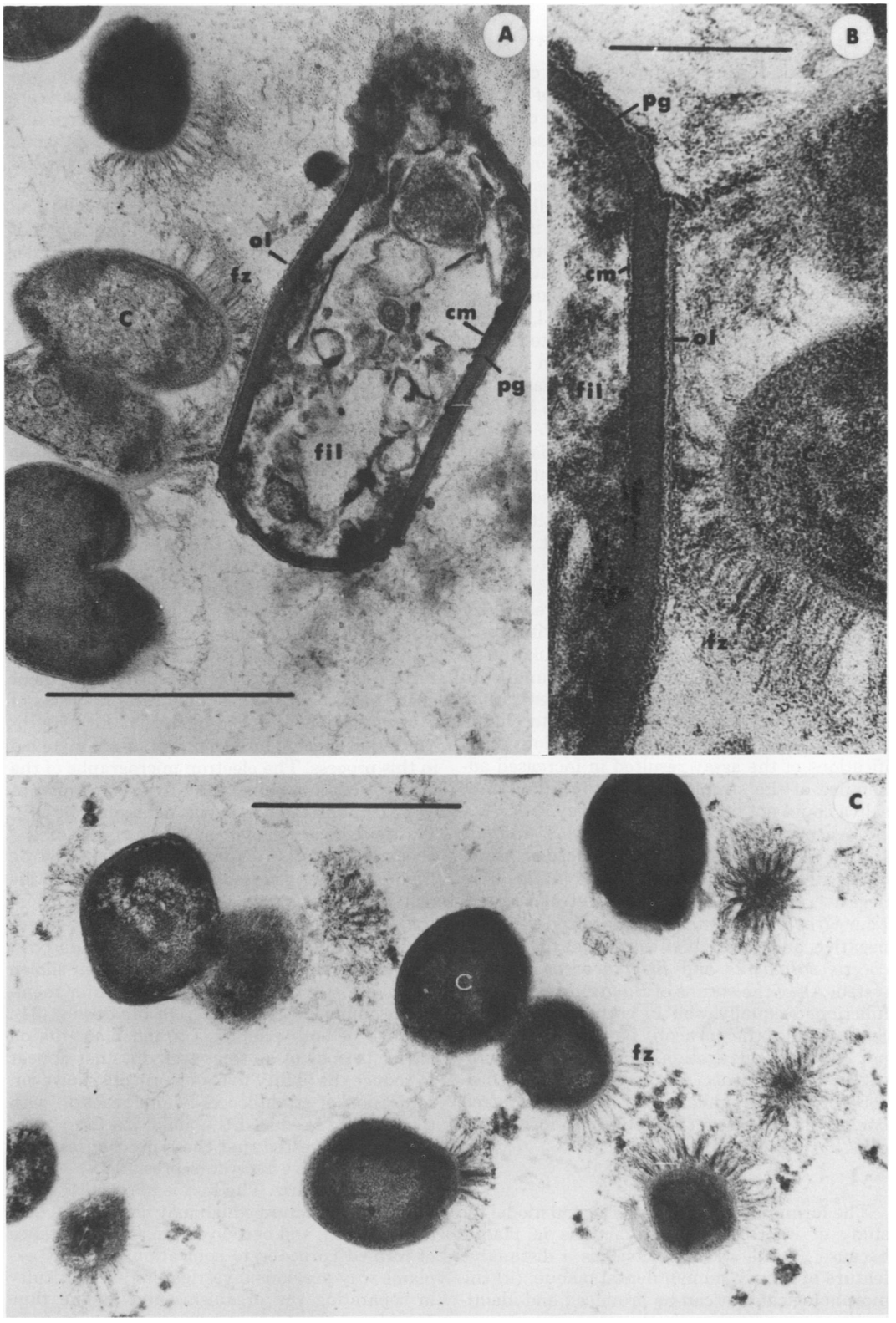

FIG. 5. *Transmission electron micrograph of thin sections of in vitro corncobs (A and B) and CC5A (C). (B) is a magnified view of (A). Bar = 1 μm (A), 0.5 μm (B), and 1 μm (C). c, Cocci; fil, filament; fz, fuzz; cm, cytoplasmic membrane; pg, peptidoglycan layer; ol, outer layer.*

found in other gram-positive cells. In contrast, the electron-opaque outermost layer of the cell more closely resembles the membrane of gram-negative bacteria. However, at the present time we do not have sufficient morphological or chemical evidence to make conclusions regarding the nature of the *Bacterionema* cell wall.

Storage of the cell suspensions on ice for 7 days did not reduce corncob formation significantly. The use of the citrate-phosphate buffer caused clumping of both cocci and filaments at pH 5 and below; in the absence of NaCl, a slight inhibition of corncob formation was noted above pH 5; phosphate buffer had no effect on corncob formation between pH 6 and 8. Variations in concentration of NaCl from 0.05 to 0.25 M in the sodium phosphate buffer (pH 6.5, 0.05 M) also had no effect on the aggregation. Variations in the phosphate molarity of the phosphate buffer between 0.005 and 0.05 M (pH 6.5) also had no effect on the corncob formations; substitution of KCl for NaCl also was without effect. In contrast, substitution of $MgCl_2$ from 0.05 to 0.25 M inhibited corncob formation up to 50%; similar inhibition was also observed in the presence of ethylenediaminetetraacetic acid. Substitution of $CaCl_2$ for NaCl showed a slight stimulation of corncob formation at 0.05 M, and the stimulation decreased with increasing $CaCl_2$ concentration; at 0.25 M the *Bacterionema* tended to clump. Attempts to use clarified saliva in various modifications of the assay resulted in increased adherence of the streptococci to the filter; thus, saliva could not be used in this assay.

Corncobs were formed only by *S. sanguis* strains CC5A, CC6, and H311. These are serotype 1 strains; nine other serotype 1 strains were negative, as were eight serotype 2 strains tested. Four *Streptococcus mitis* strains tested were also negative, as were each of the strains of *Streptococcus salivarius* and *Streptococcus faecalis* tested. All of the strains of *Bacterionema* tested functioned equally well in corncob formation. Sonication of the corncobs for up to 5 min did not cause disaggregation; however, prior sonication of streptococci for 5 min prevented corncob formation; similar treatment of *Bacterionema* did not affect their ability to form corncobs.

DISCUSSION

The formation of corncobs is a good model for study of interbacterial interactions in plaque because: (i) the structure itself is a distinctive feature of in vivo human dental plaque; (ii) the morphological unit can be produced and identified in vitro; (iii) the coccal component, *S. sanguis*, because of its ability to colonize tooth surfaces and interact with other oral bacteria, may play a pivotal role in plaque formation; and (iv) specific antisera and *S. sanguis* antigens are available for use as markers. The use of radioactive labeling for quantitating the streptococci attached to the filaments has several advantages over counting the attachment visually. First, corncobs are three-dimensional structures, and the cocci adhering at certain angles might not be visible in the microscope. Second, the number of microscope counts required in these studies would be prohibitive in terms of time and technical proficiency. Third, radioisotope counting is inherently more precise than visual methods. Although visual counts were not done, samples were routinely checked to insure that the radioactivity adhering to the membrane did represent corncob, not merely clumps of streptococci and *Bacterionema*; this was mentioned earlier as one of the advantages of the corncobs as a model of interbacterial interactions. The observation that the *Bacterionema* becomes saturated at a coccus/filament ratio of 10:1 (Klett units) is similar to that reported by others (14, 15, 22, 23, 35). Moreover, the morphological observation that the streptococci form a uniform layer over the surface of the *Bacterionema* suggests that there were a finite number of binding sites per unit surface area of *Bacterionema* for the attachment of the streptococci. Since only certain strains of *S. sanguis* have these components, our hypothesis is that specific surface receptors are involved in this process. The electron micrographs of the in vitro corncobs show structures very similar to those demonstrated in in vivo dental plaque (17). The fuzz layer, shown on the surfaces of the cocci, lacks the structural organization of true fimbriae and appears somewhat longer than the usual *S. sanguis* fuzz (16, 31). The structure of the *Bacterionema* filaments is somewhat unusual inasmuch as the organisms are gram positive in staining reactions but have been shown to have a structure resembling an outer membrane found in gram-negative organisms (34). Storage of both streptococci and filaments on ice for periods of up to a week does not appear to reduce the ability to form corncobs; however, sonication of streptococci before reaction with the filaments reduced the ability to form corncobs and suggested that these procedures inactivate or remove receptors presumably associated with the fuzz. The fuzz is apparently quite a complex structure which may contain teichoic acid, protein, and carbohydrate. The resistance of formed corncobs to sonication probably explains why previous investigators had difficulty in separating the organisms and at one time considered the cocci to be variant forms of the filaments (9, 32, 33). Indeed, cultural techniques used to isolate corncobs included micromanipu-

lation to obtain the corncobs from plaque, followed by growth under conditions which supported coccal growth but not the *Bacterionema* (21). Simple streak plates were not sufficient to separate the organisms (12). The effects of heat were not examined because this parameter was not involved in developing the assay. In addition, the effects of heat could involve denaturation, extraction, or both and thus contribute little at this point to specify the component involved in the binding reaction.

The concentration of NaCl in the assay had little effect on corncob formation; neither did the use of various molarities of phosphate buffer at pH 6.5. Replacement of NaCl by $CaCl_2$ had a slight stimulatory effect at low concentrations but increased the variation between assays. $MgCl_2$ or ethylenediaminetetraacetic acid inhibited corncob formation. These observations plus the clumping obtained with the citrate-phosphate buffer suggest that divalent cations might be important in the binding reaction. The negative counterions do not seem as important, since no differences were detected between Cl^- or HPO_4^{-2}. The stability of the corncobs to sonication suggests a firm interaction between the surfaces of the two organisms.

A comparison of those *S. sanguis* strains which form corncobs (CC5A, CC6, and H-311) and those which do not suggests that: (i) lipoteichoic acid is not solely responsible for binding, since all *S. sanguis* strains have lipoteichoic acid; (ii) the polysaccharide *b* antigen (2, 28) is not involved because no serotype 2 strains produce corncobs; and (iii) localized tufted fuzz, or fimbriae, appears to be a necessary but not sufficient condition for corncob production, since strain H-24, which also has such fuzz (13), did not produce corncobs. However, only those strains which have localized fuzz form these aggregates. Although our primary hypothesis is that an as yet unidentified surface component is responsible for corncob formation in the streptococci, it is possible that quantitative differences in one of the already identified surface components may be involved. The possibility that some inhibitor is present on the surface of streptococci not forming corncobs must also be considered. In contrast to the streptococci, the *Bacterionema* surface component appears to be found in all strains tested. At present, studies are being directed at the identification of these components in extracts of both the streptococci and the *Bacterionema*.

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